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The Role of Diet in Blood Regeneration

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ABSTRACT

This bulletin presents the results of an investigation dealing with the effects of certain nutrients and foods on the recovery of dogs from hemorrhagic anemia.

The basic synthetic ration was modified by substituting the heat-stable factors of yeast or the alcohol-soluble factors of yeast for untreated yeast; by replacing the ferric citrate with ferric ammonium citrate; and by adding ascorbic acid. In subsequent experiments a part of the synthetic diet was replaced by autoclaved wheat, beef heart, beef muscle, or beef liver; by raw liver; by thiamin and autoclaved liver; and by liver heated to various temperatures.

Criteria for assessing progress included hemoglobin and red cell volume regeneration, red cell count, and red cell size as measured by mean diameter and mean corpuscular volume.

Hemoglobin production and red cell volume regeneration were most satisfactory when autoclaved yeast was introduced into the ration immediately following a control period on the synthetic food, and were least satisfactory when autoclaved yeast or wheat was included in the diet. When some essential material from autoclaved liver was supplied, apparently reserves built up during the control period became available.

Confirming earlier studies on hemorrhagic anemia which has not been satisfactorily treated, red cell counts exceeded normal values, and did not return to the desired level until cells of normal size were produced.

Mean cell diameter and mean corpuscular volume values indicated a marked microcytosis at the beginning of each experimental period. Red cell diameter and mean corpuscular volume varied in opposite directions in many of the tests. Diameter showed the greatest increase when the diet included raw liver or liver which had not been heated above 100° C., and the greatest decrease in two series of experiments in which autoclaved liver was fed. The most striking increase in mean corpuscular volume occurred when thiamin was added to the autoclaved liver diet.

Apparently the restoration of total red cell volume and hemoglobin is governed by a different set of factors from those which operate in the return of red cell diameter or individual cell volume to pre-hemorrhagic levels.

The Role of Diet in Blood Regeneration

INTRODUCTION

HEMORRHAGIC ANEMIA occurs with sufficient frequency to constitute an important problem. In an earlier publication the authors (27) have presented a comprehensive picture of blood changes taking place in this type of anemia. The fact that conditions in this investigation could be controlled made it possible to secure a more accurate conception of the alterations in the blood associated with hemorrhages than would have been possible with human subjects. Moreover, the experimental animals, normal healthy dogs, were maintained on a uniform synthetic diet and were bled in regulated amounts.

Since recovery from hemorrhagic anemia is often largely dependent upon the nutrients available to the individual, the present investigation has been undertaken to study the effect of various foods and nutrients in restoring the blood picture to normal. In view of the limited data at present available in this field, any research evaluating foodstuffs in terms of their efficacy in blood regeneration will contribute valuable information. The measurements have centered about the erythrocyte since the earlier study showed that the most persistent changes in hemorrhagic anemia involved the total cell volume, the red cell count, the size of the individual cells, and the hemoglobin.

Specifically, the experiments reported herein have dealt with the effect upon blood regeneration (a) of the heat-stable factors and the alcohol-soluble factors of yeast in the synthetic ration, (b) of the sources of iron in the synthetic ration, (c) of the addition of ascorbic acid to the synthetic ration, (d) of the replacement of a part of the synthetic ration with food materials which supply significant portions of the iron, energy, and other dietary constituents, (e) of added crystalline thiamin during one of these dietary periods, and (f) of liver treated at various temperatures.

EXPERIMENTAL PROCEDURE

Selection of Dogs

The dogs chosen for these experiments were healthy adult animals ranging in weight from 10 to 25 kilograms. A preliminary observation period on each animal, together with a comparison of the initial values obtained for the blood factors measured in this study with the normal values previously published from this laboratory (27, 28), served as the bases for selecting suitable experimental dogs.

Basic Ration

The basic ration for all experiments described herein was the same as that previously described (27). The ingredients used were as follows:

Composition of synthetic ration

	Parts by weight	Per cent
Casein	6.30	40.25
Sucrose	4.50	28.75
Cod liver oil	0.60	3.80
Lard	3.30	21.10
Bone ash	0.25	1.60
Salt mixture	0.20	1.30
Dried yeast	0.50	3.20

Composition of salt mixture

	Parts by weight	Per cent
Sodium chloride	10.0	49.50
Calcium lactate	4.0	19.80
Magnesium citrate	4.0	19.80
Ferric citrate	1.0	4.95
Copper sulfate, crystalline	0.2	1.00
Potassium chloride	1.0	4.95

The iron and copper in this ration amount to 11.88 mg. and 3.20 mg., respectively, per 100 g. of food.

The synthetic diet was fed in amounts sufficient to maintain weight equilibrium. Although dogs were frequently kept on this ration for periods as long as nine months, no difficulties were experienced in getting the dogs to eat their food and no evidences of vitamin deficiencies were observed, such as those reported by Steenbock, Nelson, and Hart (51) for vitamin A; Cowgill (8) for thiamin; Elvehjem, Madden, Strong, and Woolley (10) for nicotinic acid; or Sebrell and Onstott (49) for riboflavin.

Development of Hemorrhagic Anemia

All animals were rendered anemic by successive bleedings from the jugular vein. Intervals of at least two days occurred between bleedings in each of which approximately one fourth of the previously measured total blood volume was taken. In those cases in which several dietary regimes were tested successively, sufficient blood was removed before each experimental period to bring the level of the cell volume to that observed at the beginning of the first experimental period. Samples for measuring the factors under consideration ranged from 30 ml. to 50 ml. for each set of determinations. They were obtained from the jugular vein in the morning before the dogs were fed.

Physical and Chemical Methods

Blood volume was measured using the dye method (Hooper, Smith, Belt, and Whipple [24]); hemoglobin by the Newcomer (36) and the Van Slyke and Neill (54) methods. Red cell counts were made with the usual technic. Mean cell diameter was determined by measuring one diameter of 200 cells in a wet film using a calibrated ocular filar micrometer. Dilutions were prepared with Hayem's solution and measurements were made as quickly as possible to prevent alteration in the size of the cells. Mean corpuscular volume was calculated according to Wintrobe's (59) formula. Hemoglobin regeneration was determined using the hemoglobin in grams per 100 ml. of blood together with total blood volume data. Cell volume regeneration was computed on the basis of increases in total cell volume from week to week throughout the test periods and from amounts of cells removed in sampling. Since the animals varied considerably in size, the hemoglobin and cell volume regenerated have been reported and compared on the basis of the amounts which have been produced per kilogram of body weight per week.

Statistical Methods

In a number of cases, where it was desired to test the significance of the difference between two means, the "t" test (11) was employed. Where an animal served as his own control, the standard error of the mean difference was calculated and its significance was determined.

EXPERIMENTAL RESULTS

Series 1—The Effect of Yeast

Originally vitamin B was thought to be a single entity but more recently it has been found to be a mixture of many compounds collectively designated as the vitamin B complex. Since yeast is regarded as an excellent source of these vitamins, the first series of experiments was planned to ascertain whether or not the heat-stable factors of yeast or the alcohol-soluble factors of yeast used in the synthetic ration are of value in blood regeneration.

Nine male and seven female dogs were used for this series of tests. Each animal was bled twice with an interval of two or three days between hemorrhages. The determinations reported were made weekly for a period of at least six weeks. Eight dogs (four males and four females) were fed the synthetic ration exclusively, thus serving as controls.

Five dogs (two males and three females) were given the basic synthetic diet modified by the replacement of the dried yeast contained therein with yeast which had been autoclaved for two hours at 120° Centigrade. This ration is designated as the synthetic diet containing the heat-stable factors of yeast.

Four of the dogs maintained on this food lost appetite. Since it seemed unwise to complicate the problem by the effects of starvation, an animal was given enough whole dried yeast (10 g. for three consecutive days) to restore appetite, after food had been refused for several days. One dog lost appetite in the fourth week after hemorrhage and again in the ninth week. Onset of anorexia occurred in two dogs in the fifth and sixth post-bleeding weeks, respectively. A fourth dog developed anorexia during the second and fifth weeks, while the remaining dog showed no loss of appetite despite the fact that he was kept on the diet for 16 weeks. He was active, alert, and in excellent physical condition throughout the entire period.

Three male dogs were fed the synthetic ration with the yeast in the basic diet replaced by an alcoholic extract prepared from a comparable amount of untreated yeast. Four hundred fifty grams of dried yeast were extracted with 1700 ml. of cold alcohol for 24 hours at room temperature, filtered with suction, washed with alcohol, and extracted a second time for 24 hours. The alcoholic filtrates and washings were concentrated on the steam

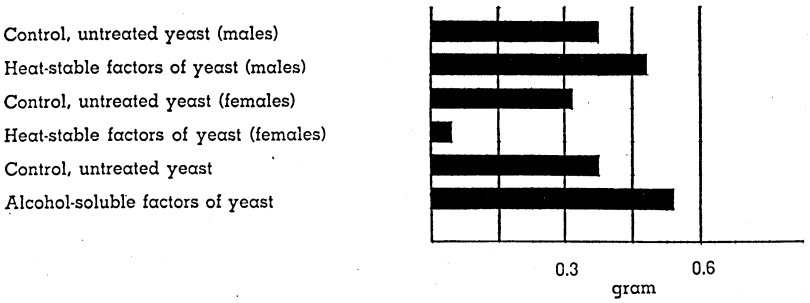
bath, evaporated on corn starch, and ground. In referring to this diet, it is described as the synthetic ration containing the alcohol-soluble factors of yeast.

In the present study one dog, after being kept on the diet three and one-half weeks, displayed the symptoms of blacktongue described by Chittenden and Underhill (7) and Goldberger and Wheeler (19), i.e., loss of appetite, foul breath, bloody diarrhea, and apathy. On the fourth, fifth, and sixth days after the onset of the condition he was given 10 g. of whole dried yeast, following which he ate all his food, was active and alert, and had no diarrhea. He remained in good condition for two and one-half weeks when the symptoms recurred. Increasing the alcoholic extract of yeast to amounts equivalent to 10 g. of whole dried yeast had no effect when given for four consecutive days, whereas 10 g. of autoclaved yeast on each of the following three days caused the symptoms to disappear. The second dog showed similar symptoms after he had been on the diet for two and one-half weeks. He refused food for a number of days, but thereafter the symptoms disappeared and did not recur although he was kept on the diet five weeks longer. The third dog remained in excellent health during the entire nine-week period during which he was under observation.

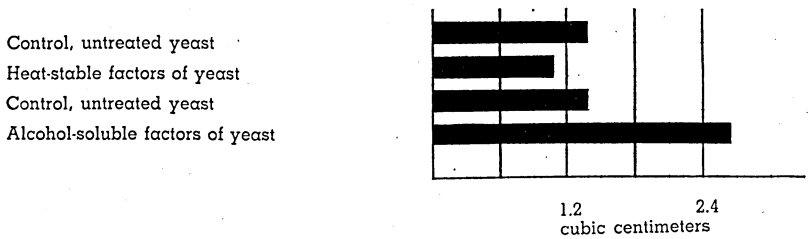
Throughout this series, in observing weekly changes on the test diets, the post-bleeding period was divided into two parts, the first two or three weeks after hemorrhage being considered a depletion period while the balance of the post-bleeding period was used as a basis for comparing the effects of different dietary regimes. During the depletion period, for example, the males and females regenerated a volume of 2.05 and 4.05 cc. of red cells per kilogram of body weight per week, respectively, whereas in the post-depletion period the values were 1.64 and 1.57 cc., respectively. During the depletion period the animals utilized such reserves of cell and hemoglobin-building materials as were contained within their bodies, and during the remainder of the period the degree of recovery was controlled by the specific diet being tested. Where no significant difference was observed between the males and the females during the post-depletion period, they have been averaged together in reporting the findings.

The results of the comparative tests using the synthetic ration containing (1) untreated yeast, (2) the heat-stable factors, and (3) the alcohol-soluble factors in yeast are shown graphically in

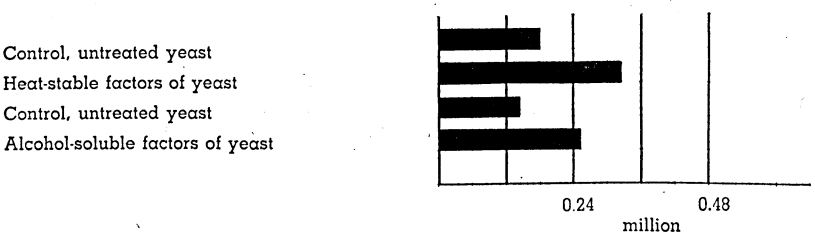
HEMOGLOBIN REGENERATED PER KILOGRAM OF BODY WEIGHT PER WEEK



RED CELL VOLUME REGENERATED PER KILOGRAM OF BODY WEIGHT PER WEEK



CHANGE IN NUMBER OF RED BLOOD CELLS PER CUBIC MILLIMETER PER WEEK



DECREASE IN MEAN CORPUSCULAR VOLUME PER WEEK

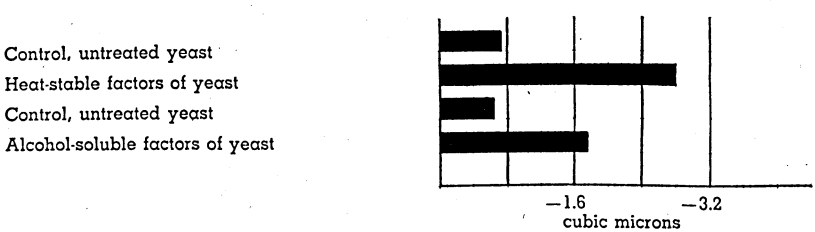


FIG. 1. THE EFFECT OF YEAST, THE HEAT-STABLE FACTORS OF YEAST, AND THE ALCOHOL-SOLUBLE FACTORS OF YEAST UPON HEMOGLOBIN AND RED CELL VOLUME REGENERATION AND UPON THE NUMBER OF RED BLOOD CELLS AND THE MEAN CORPUSCULAR VOLUME

figure 1. Hemoglobin regeneration in the control animals proceeded at rates equivalent to utilizations of 13.5 and 10.3 per cent of the iron intake[1] for the males and the females, respectively. In the dogs given autoclaved yeast, hemoglobin was formed at rates amounting to utilizations of 18.3 per cent of the ingested iron for the males and 1.6 per cent for the females. The only pair of groups between which a significant difference was found was the males and females on autoclaved yeast ($P = 0.010$). Cell volume regeneration took place at a somewhat higher rate for the control than for the test group. The mean weekly increase in red cell count was somewhat more rapid for the autoclaved yeast group than for the control group. Decreases in mean corpuscular volume were observed in both groups. The greater decrease was associated with the more rapid increase in cell counts in the test animals.

When various statistical procedures were applied to the results obtained from the dogs maintained on the synthetic ration containing untreated yeast and to those secured from animals receiving a diet containing the heat-stable factors of yeast, the number of dogs proved too small to have relationships which were significant. When these experiments are considered in the light of the findings reported by Leichsenring and Biester (27) for hemorrhagic anemia of different degrees of severity, it is evident that the performance of the group receiving the diet containing only the heat-stable factors in yeast is not as satisfactory as that of the control group of dogs. The animals on the basic synthetic ration formed a greater volume of red blood cells; they produced fewer cells, but these cells were larger and approached more nearly the size represented by a normal cell. Thus the control group may be regarded as being in more favorable condition, suggesting that heating yeast destroys some factor or factors essential in blood regeneration.

For the dogs receiving the synthetic ration containing the alcohol-soluble vitamins in yeast, hemoglobin regeneration during the post-depletion period proceeded at a rate representing a utilization of 21.2 per cent of the dietary iron compared with the 13.5 per cent in the control male dogs. In cell volume regeneration and red cell counts, the controls made smaller increases than did the test animals. Although decreases in mean corpuscular

[1] An iron value for hemoglobin of 0.34 per cent was used in computing the percentage of food iron utilized for hemoglobin regeneration.

volume were observed in both groups of dogs, the greater decrease was noted in the test animals.

It is evident that the test animals made better recoveries than did the controls as far as hemoglobin regeneration, cell volume regeneration, and increases in red cell counts are concerned, but showed a greater decrease in mean corpuscular volume. From this it is apparent that a deficiency of those factors in yeast which are insoluble in alcohol did not limit the production of hemoglobin and red blood cells in these animals.

Series 2—The Effects of Two Ferric Salts, Ascorbic Acid, and Four Food Materials

In the second series of experiments, three male dogs were used to investigate two additional aspects of the synthetic ration as well as to study the effect of replacing a part of the synthetic diet with food materials which supplied a significant portion of the iron, energy, and other dietary constituents.

These dogs were rendered anemic by five or six hemorrhages spaced at intervals of 10 to 14 days. With this number of bleedings, the reserves of the dogs appeared to be depleted. Before each experimental period, enough blood was removed to reduce the total cell volume to the level observed at the beginning of the control period. During the time in which various foods and vitamin supplements were being tested, measurements were made at weekly intervals as well as at the beginning and at the end of the experimental period. Observations extended over three weeks with the exception of the ascorbic acid and the raw liver periods, which continued for two weeks.

Since some differences were observed in the responses of individual animals making up the several groups of dogs compared in Series 1, it seemed desirable to eliminate this source of variation in this and subsequent series of tests by having each animal serve as his own control. At the beginning of the series, therefore, a three-week test period in which the synthetic ration, only, was fed has been introduced. The first two weeks of this period were used in making comparisons with the ascorbic acid and raw liver experiments.

In the second period the ferric citrate of the basic synthetic diet was replaced by ferric ammonium citrate in the amount needed to supply an equivalent amount of iron. During the third period the dogs were again given the basic synthetic ration supplemented once each week by the intravenous administration of

200 mg. of ascorbic acid dissolved in physiological salt solution and very carefully neutralized with sodium bicarbonate. In order to test whether food sources of iron would be more valuable for hemoglobin and cell volume regeneration than the iron of the synthetic ration, a portion of the latter was replaced by various foods. In the fourth period 250 g. of raw beef liver replaced an equicaloric amount of the basic synthetic diet. The iron content of the synthetic ration used was adjusted so that the daily iron intake was the same as in the control period.

This group of dogs was kept throughout the summer and maintained on the basic synthetic diet, at the end of which all animals appeared to be in excellent condition. They were used again for a second year of research during which a similar plan was followed. A control period of three weeks preceded four three-week test periods during which equicaloric portions of the synthetic ration were successively replaced by cooked ground whole wheat (60 g.), cooked beef muscle (round) (250 g.), cooked lean beef heart (250 g.), and cooked beef liver (250 g.). A fifth period of two weeks followed during which 250 g. cooked beef liver plus 250 I.U. thiamin were used. The wheat was heated at 120° C. for two hours, and the meat was cooked in lots of 10 or 12 pounds at 120° C. for 20 minutes per pound. Throughout this entire series of experiments, the iron in the synthetic diet was adjusted so that the total amount fed was held constant. Figures 2, 3, and 4 present a comparison of the recoveries made in hemoglobin and cell volume regeneration, red cell count, cell diameter, and mean corpuscular volume during the various test periods. The histograms in these figures are arranged in the order in which the diets were fed.

The relative insolubility of ferric citrate in the basic ration suggested the desirability of comparing the degree of recovery when this compound served as the source of iron with that noted when the more soluble ferric ammonium citrate was used. In order to assure that no additional amounts of iron were derived from other components of the diet, a sample of the prepared food was analyzed for "available" iron using the dipyridyl method. The results gave good agreement with the computed value for iron in the synthetic ration.

During the ferric citrate period hemoglobin was regenerated at a mean rate which constituted a utilization of 17.0 per cent of the iron intake, while during the ferric ammonium citrate period, 17.2 per cent of the iron fed was used for this purpose. From these

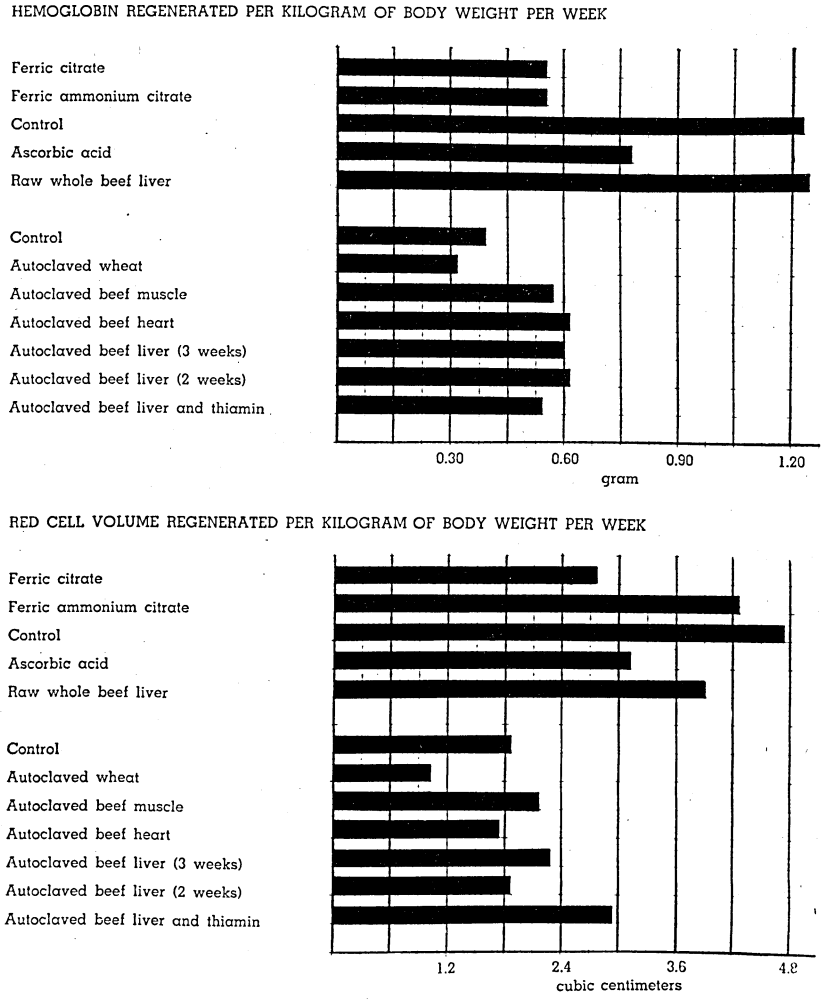


FIG. 2. THE EFFECT OF TWO FERRIC SALTS, ASCORBIC ACID, AND SEVERAL DIFFERENT FOOD MATERIALS UPON HEMOGLOBIN AND RED CELL VOLUME REGENERATION

results it is apparent that the iron in these compounds is used by dogs with equal facility for hemoglobin production. Mean cell volume regeneration was appreciably higher with ferric ammonium citrate as the source of iron than during the ferric citrate period. Mean weekly increases in the number of red blood cells also were slightly greater during the ferric ammonium citrate period when compared with the ferric citrate period. In the ferric citrate period, decreases occurred in both mean cell diameter and

in mean corpuscular volume. The ferric ammonium citrate period was characterized by a very small increase in cell diameter and a decrease in mean corpuscular volume. Since considerable data had been accumulated in this laboratory using a synthetic ration containing ferric citrate and since the results of the comparative tests on ferric ammonium citrate and ferric citrate are quite similar, the synthetic ration with ferric citrate was selected as a control for the tests in this series.

Dogs generally are said not to require a dietary source of ascorbic acid and therefore none was included in the synthetic ration. It is possible, however, that massive hemorrhage may sufficiently increase the need for this vitamin so that its lack may constitute a limiting factor in blood regeneration, since it has been claimed by a number of workers that ascorbic acid is required for all stages in the maturation of the red blood cells. Consequently, the effect of the intravenous injection of measured amounts of ascorbic acid was studied. The value of raw liver for blood regeneration was also investigated.

Hemoglobin regeneration during the first two weeks of the control period showed a utilization of 37.0 per cent of the ingested iron compared with 23.5 per cent during the ascorbic acid period. Mean cell volume regeneration was greater during the control period than during the ascorbic acid period. These findings were associated with corresponding increases in red cell counts. Identical decreases in mean cell diameter were observed during both periods, while decreases in mean corpuscular volume in the control period exceeded those in the ascorbic acid period. From these data it is evident that the addition of ascorbic acid does not in any way facilitate blood regeneration in dogs. It appears that this species does not require ascorbic acid or is able to synthesize this vitamin in amounts adequate to meet its needs even in severe hemorrhagic anemia.

The first food tested in this series was raw beef liver. It was fed in large pieces mixed with a portion of synthetic food. Comparing the hemoglobin regenerated in the control period with that in the raw liver period shows similar utilizations (37.0 and 37.2 per cent) of the ingested iron. Appreciable quantities of cells were regenerated during both periods with parallel increases in the red cell counts. In the control period, the mean red cell diameter remained essentially unchanged but it showed a marked increase when raw liver was fed. The weekly decrease observed in mean corpuscular volume in the control period was greater than

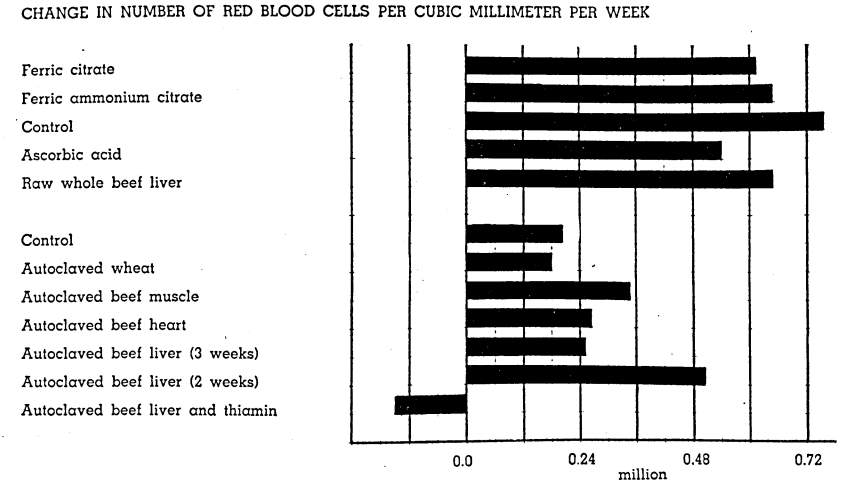


FIG. 3. THE EFFECT OF TWO FERRIC SALTS, ASCORBIC ACID, AND SEVERAL FOOD MATERIALS UPON THE CHANGE IN THE NUMBER OF RED BLOOD CELLS PER CUBIC MILLIMETER PER WEEK

that noted in the raw liver period. It is evident that feeding raw liver affects the size and shape of the red blood cells. The diameter increases and the volume of the individual cell decreases less than it does during the control period. When raw liver is fed for a two-week period the red blood cells approach the normal in number, diameter, and individual cell volume.

Following this test period, the three dogs were kept on the synthetic diet for three months. Since weight could be maintained at this time by feeding the ration at a somewhat lower level, a second control period was introduced before testing wheat, beef muscle, beef heart, beef liver, and beef liver plus thiamin. All foods were cooked in an autoclave before mixing with a portion of the synthetic ration. In the last experiment, the thiamin was added after the beef liver had been heated.

The observed rate of hemoglobin regeneration during the control period of the second year of the series represented an iron utilization of 14.4 per cent in contrast to 11.3 per cent when wheat replaced a portion of the synthetic diet. Hemoglobin production was almost identical when 250 g. beef muscle, beef heart, or beef liver replaced a portion of the synthetic diet. Utilizations of 21.0, 23.0, and 22.1 per cent, respectively, of the iron in the food were found. Trends in cell volume regeneration followed those for hemoglobin.

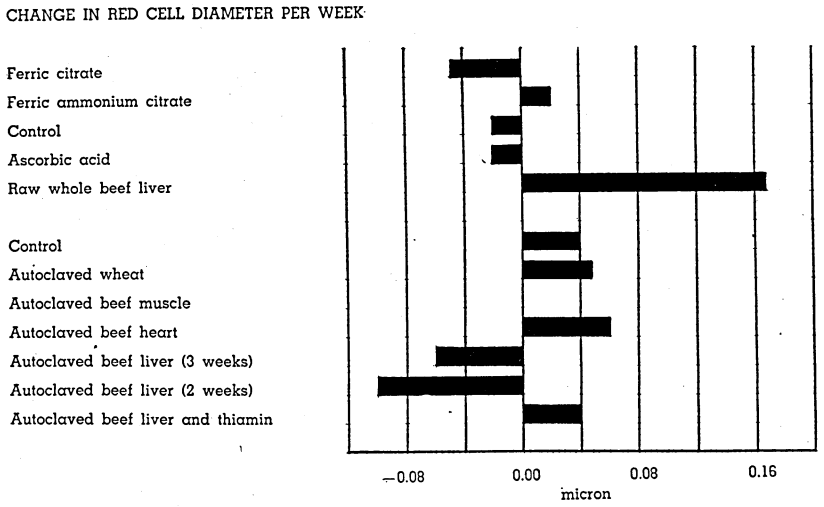


FIG. 4. THE EFFECT OF TWO FERRIC SALTS, ASCORBIC ACID, AND SEVERAL DIFFERENT FOOD MATERIALS UPON THE CHANGE IN THE SIZE OF THE RED BLOOD CELLS AS SHOWN BY DIAMETER AND MEAN CORPUSCULAR VOLUME MEASUREMENTS

Similar increases in red cell counts were observed for the control and the wheat periods, followed by greater increases in the beef muscle, beef heart, and beef liver periods. Increases in mean cell diameter of essentially the same magnitude were found in the control, wheat, and beef heart periods. During the beef muscle period, cell diameter remained unchanged, while in the beef liver period a decrease was noted. In mean corpuscular

volume, weekly increases were observed in the control, beef muscle, and beef heart periods, while decreases were noted in the wheat and beef liver periods.

On the whole, the response of the dogs to the autoclaved liver supplement at the end of the second year was less favorable than that found with raw liver, a year earlier. On the assumption that part of the thiamin might have been destroyed in autoclaving the liver, 250 I.U. of thiamin chloride were added daily to the ration which consisted of the synthetic food and autoclaved beef liver. Weekly hemoglobin regeneration in the autoclaved whole liver period (based on a two-week period) represented a utilization of 22.5 per cent of the ingested iron, whereas in the autoclaved liver-thiamin period a utilization of 20.0 per cent was found. The superiority of the liver-thiamin period over the autoclaved liver period is shown both by the comparative changes in cell diameter and mean corpuscular volume. For the latter the difference approached statistical significance ($P=0.052$). Although thiamin apparently does not influence the regeneration of hemoglobin, it appears to increase the total volume of cells produced due to an increase in both the diameter and volume of the individual cells.

In the test periods involving various foods in Series 2, the poorest performance was observed during the wheat period and the best during the raw liver period. Intermediate responses were noted for autoclaved beef muscle, beef heart, and beef liver, as well as beef liver supplemented with thiamin, with the exception that mean corpuscular volume showed the greatest improvement in the thiamin period.

Series 3—The Effect of Liver Heated at Different Temperatures

The observations in Series 2 indicated that raw liver was more effective than autoclaved liver in producing cells which were normal in number and which approached normality in diameter. Consequently Series 3 was undertaken with a view of testing the efficacy of liver heated at various temperatures for blood regeneration.

Five male dogs were bled five times in order to deplete body reserves of blood-building materials. The progress made on the different dietary regimes has been based upon the means for values for the several blood factors obtained on two consecutive days at the beginning and at the end of the test periods. Before each period, with the exception of the raw whole liver period,

enough blood was removed to bring the level of red cell volume to that observed at the beginning of the first experimental period.

In the studies involving this group of dogs, a control period of three and one-half weeks on the basic synthetic ration was followed by test periods of two weeks, during which an equicaloric portion of the basic synthetic ration was replaced by 135 g. of liver for one dog and by 200 g. of liver for the other dogs which were larger animals. The total daily iron intake was held constant. During the successive experimental periods the liver used was ground, mixed with 100 ml. distilled water, and heated for 20 minutes at 120°, 100°, 80°, and 60° centigrade. With the exception of the 120° C. liver period, the material was stirred continuously to insure a more uniform temperature throughout the mass. In the last two periods, raw ground liver which had been stirred in the same manner as the cooked liver, and raw liver in large pieces supplemented the synthetic ration. Figures 5 and 6 show the results of these experiments.

The difference between the hemoglobin regenerated during the control period and the autoclaved liver period was statistically significant ($P=<0.016$). Utilizations of 10.7 and 40.9 per cent of the ingested iron, respectively, were noted. Compared with the autoclaved liver period, progressively less hemoglobin was produced during the boiled, the 80° and 60° C. liver periods, and the ground raw liver period. The amounts formed corresponded to utilizations of 31.6, 20.2, 18.0, and 13.2 per cent of the iron intakes, respectively. When raw whole liver was fed during the last period in the series, a somewhat improved utilization of iron (22.7 per cent) occurred. When tested statistically a significant decrease from the rate of hemoglobin regeneration in the autoclaved liver period was noted in the 60° C. liver period ($P=<0.007$), and in the raw whole liver period ($P=>0.044$). The decrease observed in the raw ground liver period approached significance ($P=0.054$).

The volume of cells produced during the autoclaved liver period also was significantly greater than that observed during the control period ($P=>0.030$). Statistically significant decreases were observed during the 80° C. liver period ($P=<0.027$) and in the 60° C. liver period ($P=<0.016$) when compared with the autoclaved liver period. The volume of cells formed during the boiled liver period was midway between the values observed in the autoclaved and 80° C. periods, whereas those for the raw ground and the raw whole liver periods were similar in magnitude to the 60° C. period.

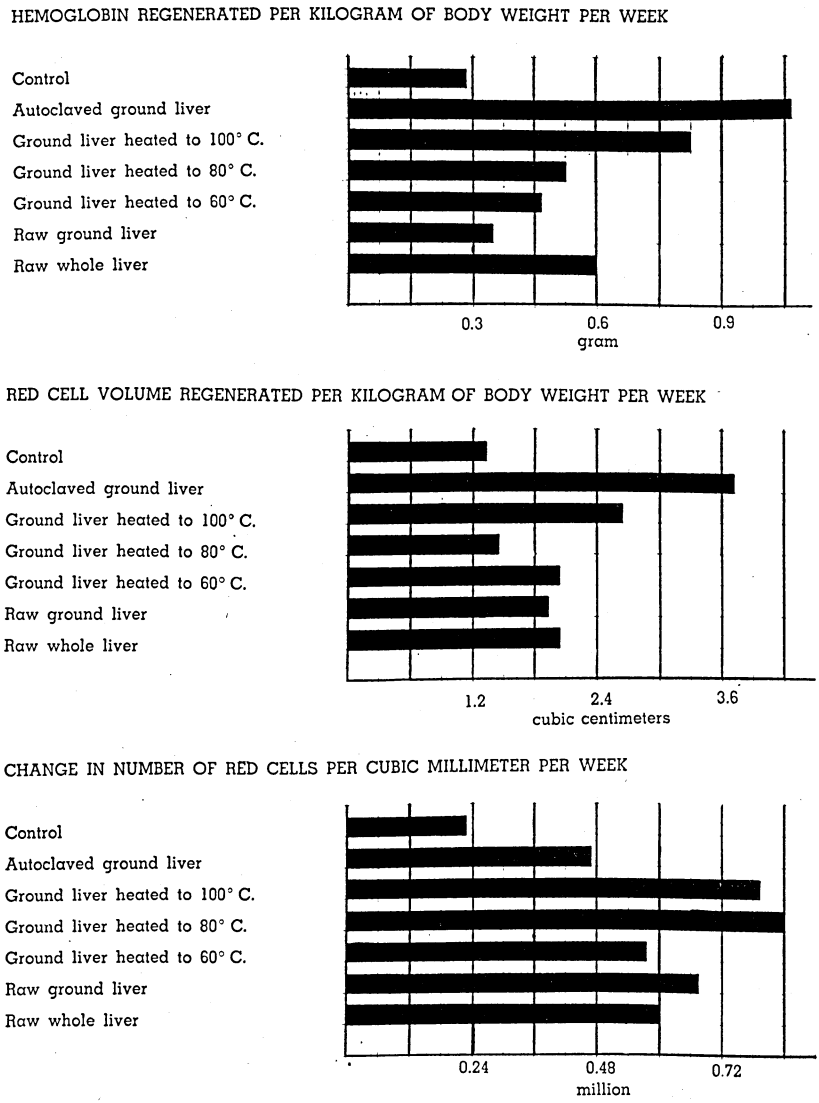


FIG. 5. THE EFFECT OF HEATING LIVER TO DIFFERENT TEMPERATURES UPON HEMOGLOBIN AND RED CELL VOLUME REGENERATION AND UPON THE NUMBER OF RED BLOOD CELLS

The more rapid increase in hemoglobin and cell volume production during the autoclaved liver period as compared with the control period was associated with a somewhat accelerated formation of red blood cells. Mean weekly increases in red cell counts occurred in all periods. The smallest increase was found

in the control period, and the greatest in the boiled and 80° C. liver periods. Intermediate values were noted in the remaining tests of the series.

Weekly decreases were observed in the mean cell diameter during the control and autoclaved liver periods. In each of the succeeding liver periods, however, a small increase in cell diameter occurred. The change in the boiled liver period was significantly different from that found in the autoclaved liver period ($P = > 0.023$). A significant increase in cell diameter was also observed in the raw whole liver period ($P = < 0.014$). Although the mean increases in cell diameter during the 60° C. and the raw ground liver periods were of the same magnitude as in the raw whole liver period, owing to greater individual differences in the responses of the animals, the differences were not significant.

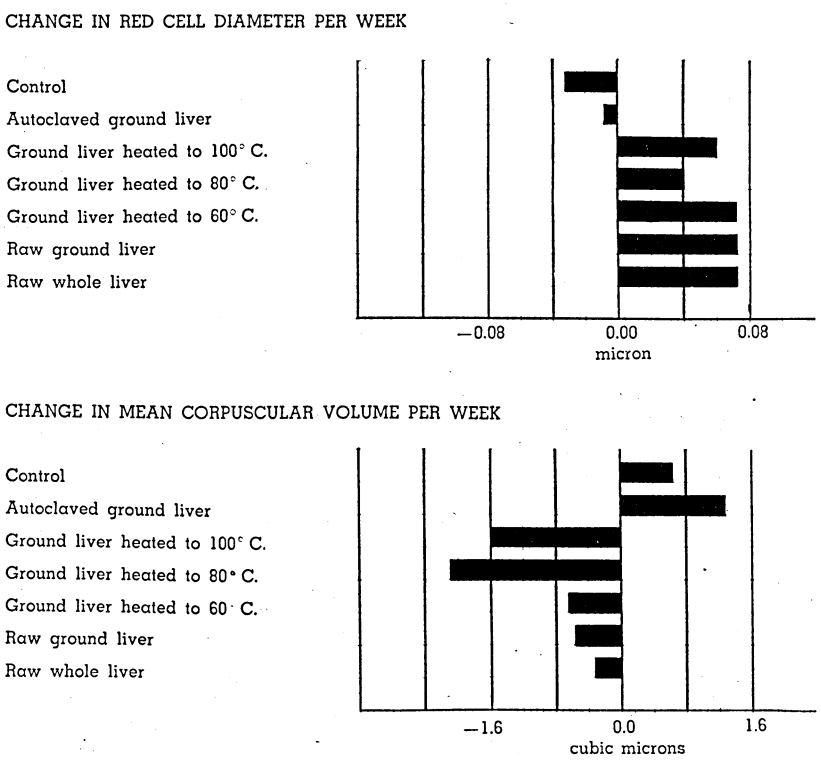


FIG. 6. THE EFFECT OF HEATING LIVER TO DIFFERENT TEMPERATURES UPON THE CHANGE IN THE SIZE OF RED BLOOD CELLS AS SHOWN BY DIAMETER AND MEAN CORPUSCULAR VOLUME MEASUREMENTS

Changes found in mean corpuscular volume were the reverse of those noted in cell diameter. Whereas decreases were observed in cell diameter in the control and autoclaved liver periods, increases occurred in mean corpuscular volumes. The increases found in cell diameter in all succeeding periods were associated with a wide range of decreases in mean corpuscular volumes. The greatest decrease, that found for the 80° C. liver period, when compared with the response to autoclaved liver proved to be significantly different ($P=<0.044$).

Consideration of the increments in the number of red blood cells in relation to the size of the cells formed shows that cell diameter decreases and mean corpuscular volume increases when less than 500,000 cells per cubic millimeter are added to the blood each week. The reverse is true when the weekly increases ranged from 560,000 to 840,000 cells. The values shown in figure 5 demonstrate the increased production of cells resulting from a specific dietary regime, but give no indication of the absolute numbers of erythrocytes per unit of blood. In these experiments, the absolute levels of cells increased with each succeeding period and exceeded the normal level with the exception of the initial control period and the count made at the beginning of the autoclaved liver period. At the end of the final test period the mean red cell count was 10.6 millions per cubic millimeter (range 10.0 to 11.6 millions) in contrast with a mean normal value of 6.2 millions for these animals.

Taken by themselves, large increases in the number of erythrocytes suggest excellent progress toward recovery. Such findings, when associated with subnormal values for hemoglobin, hematocrits, cell diameter, and mean corpuscular volume, reemphasize the need for a combination of measurements for assessing the improvement made by an anemic individual.

In Series 3, the most satisfactory responses, measured in terms of hemoglobin and red cell volume regeneration or individual cell volume, were obtained with the synthetic ration supplemented with autoclaved liver. On the other hand, red cell diameter measurements showed the poorest response with autoclaved liver. In Series 2 the red cell diameter was affected in a similar manner during the autoclaved liver period. Apparently changes in red cell diameter are not governed by the same factors which control hemoglobin production or the total and individual cell volume.

DISCUSSION

The Effect of Diet upon Hemoglobin and Red Cell Volume Regeneration

The findings in the present study show that, for dogs rendered anemic by bleeding, ferric citrate is as satisfactory for hemoglobin production as the more soluble ferric ammonium citrate. However, during the latter period the greater mean weekly increase in red blood cells, associated with a smaller decrease in individual corpuscular volume, resulted in the production of a somewhat larger total volume of cells.

Whipple and Robscheit-Robbins (57) found no difference in utilization by their standard anemic dogs when the iron was given in ferrous or ferric compounds. Murphy (35), on the other hand, reported ferric ammonium citrate more effective than ferric citrate in cases of secondary anemia in human subjects, while Lottrup (30) observed that ferric preparations had no effect, whereas ferrous salts caused a rapid increase in hemoglobin percentage in simple anemia of children. Hahn (20) suggested that it is not the form in which the iron is ingested but some unknown intestinal factor which determines its absorption and utilization.

There are many types of anemia resulting from a variety of causes. In clinical practice, changes in hemoglobin level and in the red cell count are used as criteria for judging the status and the progress made by anemic individuals. In experimental work, additional factors have been measured. These include, for example, red cell hematocrit, total blood volume, cell diameter, and total or individual cell volume. The fundamental differences in the anemias, together with the criteria selected for assessing progress, may account for some discrepancies in the literature.

In the wheat test period in the present investigation, although the mean weekly increase in red cell counts was greater than during the control period, it was associated with a decrease in individual red cell volume as contrasted with an increase during the control period, resulting in the production of a smaller volume of cells. Hemoglobin regeneration during the wheat period was also less than in the control period.

Free and Bing (16) found that the iron in wheat reacting with dipyridyl ranged from 73 to 88, with an average of 81 per cent. In studies on anemic rats they found that the iron of wheat was as available as an equal amount of ferric chloride, 50 per cent of the iron in each case being retained by the animal.

Although Rose and co-workers (45, 46, 47) reported excellent hemoglobin production with whole wheat in rats with nutritional anemia, the better results obtained with the natural cereal as contrasted with those secured when the ash only was added to the diet led them to conclude that natural cereals contain some substance other than iron or copper which stimulates regeneration of hemoglobin. In a later study (53) they obtained better hemoglobin regeneration in rats when predigested wheat was fed than when the whole grain was given, indicating that completeness of digestion was a factor affecting hemoglobin production. Although the wheat used in the present study was ground and thoroughly cooked, it is possible that the extent to which dogs digest autoclaved wheat influenced the findings.

In the beef muscle period, the mean increase in red cell counts was considerably greater than during the control period. This was associated with a somewhat larger increase in individual corpuscular volume, resulting in a greater cell volume production. Hemoglobin regeneration also was slightly better in the beef muscle than in the control period.

The mean weekly increase in red cell counts during the beef heart period was likewise slightly greater than during the control period. Although an increase in individual corpuscular volume was again noted, total cell volume regeneration was slightly less than in the control period, an apparent inconsistency. A little more hemoglobin was produced in this period than in the control period, but slightly less than in the beef muscle period.

In Series 2 and 3 in which the value of autoclaved liver for blood regeneration was tested, increases in red cell counts, hemoglobin, and total cell volume were appreciably greater during the liver period than during the control period. Since the total iron intake was kept constant throughout these experiments, the greater recovery made during the liver period must be attributed to some substance in liver other than the iron.

Throughout the three series, the volume of cells regenerated reflects the increase in the number of erythrocytes as well as the size of the cells produced. Hemoglobin values frequently parallel rather closely those for cell volume, suggesting that hemoglobin regeneration may have been limited by cell volume production.

The findings on liver are in agreement with those of Hahn and Whipple (21) who reported that its iron content is not wholly responsible for its favorable effect in hemorrhagic anemia. Frost, Potter, Elvehjem, and Hart (17), working with dogs fed on milk

exclusively and rendered anemic by repeated bleedings, were unable to demonstrate that whole dried liver gave any better recoveries than did a combination of inorganic iron and copper. In a more recent study, however, Frost, Spitzer, Elvehjem, and Hart (18) found that whole dry liver or liver extract overcame the inhibitory effect of cobalt on the hematopoietic response to iron and copper feeding observed in dogs rendered anemic by hemorrhage. In the studies of Robscheit-Robbins and Whipple (43) beef heart produced a favorable but not striking increase in hemoglobin as contrasted with beef liver, while beef muscle was less potent than beef heart. According to Sherman, Elvehjem, and Hart (50), the iron content of beef heart and beef liver is 60 per cent available, while that of beef muscle is 50 per cent available. Oldham (37) reported that heating beef muscle makes the iron as available for hemoglobin synthesis as the iron in ferric chloride.

If recovery in these experiments were determined only by the available iron in the diets, it would be expected that the best progress in Series 2 would have been observed in the control period, followed by wheat, beef heart, beef liver, and beef muscle in the order named. In the present study, using hemoglobin production as a criterion, the iron of the raw whole liver was as satisfactorily utilized as that of the ferric citrate. When autoclaved liver was fed in Series 3, the percentage of iron utilized was better than in the control period. Evidently some factor or factors, in addition to iron, influenced hemoglobin regeneration.

This hypothesis is further borne out by the results obtained in Series 3. The greatly improved iron utilization which occurred when autoclaved liver was fed as contrasted with that observed in the control period indicates that hemoglobin production was limited in the control period by the lack of, or the inadequate supply of, some needed substance which was furnished by autoclaved liver. The tendency for hemoglobin regeneration to decrease with each succeeding liver period supports the view that a second limiting factor entered into the picture. A possible explanation would be that during the control period hemoglobin production was limited by the lack of some essential factor other than iron, resulting in a surplus of absorbed iron which was stored in the animal body. The autoclaved liver in the succeeding period supplied this essential substance, resulting in rapid hemoglobin regeneration due to the relatively large amount of available iron in the body stores. The decrease in the hemoglobin formed in succeeding liver periods resulted from the gradual de-

pletion of these iron reserves. Copper was supplied in adequate amounts in the synthetic ration in the present study, and hence limited hemoglobin production could not have been due to a deficiency of this element.

In addition to the effect of adequate iron in an available form, the importance of sufficient protein of high biological value for blood regeneration has been emphasized by studies such as those of Heath and Taylor (23), Hahn and Whipple (21), Pearson, Elvehjem, and Hart (40), and Bethell, Gardiner, and MacKinnon (3). That protein was not the limiting factor in the present study would seem apparent since the daily protein intake of the animals ranged from 50 to 60 g., 50 to 80 per cent of which was supplied from beef liver and the balance from casein. Furthermore, in Series 3 the gradual decrease in hemoglobin and cell volume production in spite of continued liver feeding would argue against the protein intake being the limiting factor.

The importance of ascorbic acid for blood formation has also been brought out as the result of investigations conducted by Mettier and Chew (33), Rohmer, Bezssonoff, Schneegans-Hoch, and Sacrez (44), and Aron (1) who report the development of anemia in scorbutic guinea pigs and its cure with the addition of ascorbic acid to the diet. In humans, anemia associated with scurvy has been reported to respond to treatment with doses of ascorbic acid by Dunlop and Scarborough (9), and Parsons and Smallwood (39). In the present study, no evidence was secured that the administration of ascorbic acid in any way facilitated recovery from hemorrhagic anemia in dogs. When the number of red blood cells or the hemoglobin and red cell volume regenerated are used as criteria, better recoveries were observed during the corresponding control period than during the ascorbic acid period. Either these animals do not need ascorbic acid or they are able to synthesize it in adequate amounts for these purposes.

The Effect of Diet upon Red Blood Cell Size

The changes in the size of the erythrocytes are shown by alterations not only in cell diameter but also in mean corpuscular volume. That the diameter and the volume of the cell may vary independently has been reported by Schalm (48). He further states that "no conclusions may be drawn concerning the one magnitude from the location of the other." According to Vaughan and Goddard (55) cell volume may be taken as a measure of cell size in certain forms of anemia (Addisonian pernicious anemia

and idiopathic hypochromic anemia), but it cannot be so taken in all anemias. Mogensen (34) suggests that these two methods for determining cell size should be looked upon as complementary and not competing. In the present investigation, similar observations have been made. In the autoclaved liver period of Series 3, the slight decrease in mean cell diameter was associated with an increase in mean corpuscular volume. In each of the succeeding periods, however, a small increase in cell diameter was noted and a decrease in mean corpuscular volume. In Series 2 in some of the experiments, the diameter and the volume of the cell varied in the same direction as is best shown in the autoclaved liver and thiamin period, in which a small increase in cell diameter was associated with a marked increase in mean corpuscular volume. In other experiments, these values varied in opposite directions as demonstrated most markedly in the raw liver period, in which a pronounced increase in cell diameter was associated with a decrease in mean corpuscular volume. The red cells must have become thinner as a result of the changes noted. The physiological significance of this alteration in size and shape is not known.

Jolly (25) suggested that microcytosis is the result of rapid cell production. Such a relationship is well illustrated in Series 1 in which small increases in the number of blood cells are associated with small decreases in mean corpuscular volume, whereas large increases in cell counts accompany large decreases in mean corpuscular volume. The best illustration of this interrelationship in the remaining series is found in the autoclaved liver period in Series 2 and in the boiled and 80° C. liver periods in Series 3.

Microcytosis, as evidenced by abnormally high red cell counts and abnormally small cells, has been observed consistently in this laboratory in hemorrhagic anemia experiments of long duration (27, 29), and has been reported by a considerable number of other workers: Wintrobe (58, 60, 61); Price-Jones (41, 42); Wintrobe and Beebe (62); Castle and Minot (6); Sturgis (52); Bethell, Isaacs, Goldhamer, and Sturgis (4); Fowler and Barer (15). Such has also been the case in this investigation in which increased red cell counts are reported. By referring to figures 3, 4, 5, and 6 it will be seen, however, that increases in the number of cells are occasionally accompanied by increases in mean corpuscular volume or by relatively small decreases.

Witts (63) and Price-Jones (42) suggested that the microcytosis of chronic hemorrhagic anemia is the result of iron deficiency. Parsons, Hickmans, and Finch (38) developed a hypochromic

microcytic anemia in young rats on an iron-deficient diet. In their animals the cells were restored to normal size by the administration of adequate amounts of iron with a trace of copper. Hawksley (22) stated that microcytosis occurs in copper-deficiency anemia, and that the mean red cell diameter returns to normal with copper therapy.

Reference has already been made to the trends in cell diameter and mean corpuscular volume in the experiments dealing with the effects of temperature on the value of liver for hemoglobin regeneration in Series 3. Since the iron and copper intakes were held constant throughout the series, the changes noted cannot be attributed to differences in the amounts of these elements fed. As previously suggested some constituent of liver may make available reserves in the body of essential hemoglobin-building material. Likewise there is great variability in the number and size of cells produced in Series 1 and 2 in which the amounts of copper and iron fed are not varied. Published data on the availability of iron in foods do not explain the results obtained.

Parsons and Smallwood (39) observed a slight microcytosis in anemia associated with scurvy. They believe that ascorbic acid is needed for all stages of maturation of the red blood cells. Barron and Barron (2) have also postulated that ascorbic acid is a regulator of red blood cell production. It is generally conceded that dogs do not require a dietary source of this vitamin. From this it might be inferred that microcytosis could not result from a shortage of ascorbic acid in this species. However, the possibility of a deficiency of this vitamin under the stress of repeated severe hemorrhages was investigated in Series 2. The intravenous injection of ascorbic acid, carefully neutralized with sodium bicarbonate, did not bring about improvement in cell size as measured either by cell diameter or mean corpuscular volume.

Fouts and others (12, 13, 14) reported the production of an anemia associated with microcytosis and hypochromia in puppies and adult dogs maintained on a synthetic diet deficient in pyridoxine. These conditions were remedied by the restoration to the diet of the missing factor, either as the pure crystalline compound or as a constituent of rice bran extract. The amount of pyridoxine required for protection against microcytic hypochromic anemia is greater than that needed for optimum growth in dogs, according to McKibbin, Madden, Black, and Elvehjem (31). Kark and associates (26) found no improvement in anemic patients even when large doses of pyridoxine were given.

Borson and Mettier (5) observed that synthetic vitamin B_6 relieves the hypochromic microcytic anemia produced in dogs deficient in this factor and that an adequate supply of the non-adsorbable fraction of the vitamin B complex is necessary for the complete disappearance of this anemia. McKibbin, Schaefer, Frost, and Elvehjem (32) have reported that pyridoxine therapy brings about an immediate stimulation in blood formation in anemia due to a deficiency of this vitamin, followed by a lag which is overcome by the addition of liver extract to the ration.

In curative experiments after microcytic anemia had developed, Fouts and his associates (13) as well as Borson and Mettier (5) allowed 60 micrograms of pyridoxine daily per kilogram of body weight for adult dogs, whereas McKibbin and others (32) administered 100 micrograms. On the basis of Waisman and Elvehjem's (56) food assays for pyridoxine, in the current study the dogs receiving raw liver had from 75 to 90 micrograms of this vitamin per kilogram of body weight daily, not including that obtained from yeast. It will be recalled that in these experiments (see figures 4 and 6, Series 2 and 3) that although increases in cell diameter were noted, mean corpuscular volume showed a decrease. It is recognized that autoclaving may destroy a portion of the pyridoxine in foods. In the autoclaved liver experiments both cell diameter and mean corpuscular volume decreased in Series 2, whereas in Series 3 the volume increased. It would appear that the failure of the cells to return to normal size must be attributed in part to factors other than the pyridoxine content of the diet. Since Waisman and Elvehjem (56) found that liver is an excellent source of riboflavin, pantothenic acid, and nicotinic acid, the extent to which any of these vitamins may be involved in restoring cells to normal size is also problematical.

These authors report that thiamin assays indicate a loss of this vitamin in most cooking processes, the degree of destruction being influenced by the temperature and the length of heating. In the research reported herein in Series 1, the mean volume of the individual cells was more adversely affected during the autoclaved yeast period than during the control period. Furthermore, considering both diameter and volume, the dogs on autoclaved liver performed less satisfactorily than those on raw liver. When thiamin was added to autoclaved liver in the last period of Series 2 both the diameter and mean corpuscular volume increased. These experiments indicate the desirability of further investigation of the role of thiamin in blood regeneration.

SUMMARY

1. This bulletin presents the results obtained in studying the effects of various nutrients and foods upon hemoglobin and red cell volume regeneration, cell count, cell diameter, and mean corpuscular volume.

2. Dogs fed a synthetic ration containing the heat-stable factors in yeast made less satisfactory recoveries from hemorrhagic anemia than did the control group of dogs on a diet with untreated yeast.

3. The recoveries of the animals on the synthetic ration containing only the alcohol-soluble factors in yeast were as satisfactory as those of the control dogs.

4. Ferric citrate and ferric ammonium citrate when incorporated in a synthetic ration were of equal value in hemoglobin regeneration.

5. The administration of ascorbic acid to dogs receiving a synthetic ration did not facilitate recovery from hemorrhagic anemia.

6. When a portion of the basic ration was replaced by equicaloric amounts of various foods in Series 2, the best recovery was obtained with raw liver and the poorest with autoclaved wheat. Intermediate responses were noted for autoclaved beef muscle, beef heart, and beef liver, as well as beef liver supplemented with thiamin, except that mean corpuscular volume showed the greatest improvement in the thiamin period.

7. In the series in which liver heated to various temperatures was fed, the most satisfactory responses for hemoglobin and red cell volume regeneration and for individual cell volume were obtained with the synthetic ration supplemented with autoclaved liver. This test period immediately followed the control period, suggesting that the liver furnished some factor which made possible the utilization of body reserves of iron or some essential material which was gradually depleted in the subsequent periods.

8. The red cell diameter measurements showed the poorest response during the autoclaved liver period in both series in which this food was tested, indicating that changes in cell diameter are not governed by the same factors which control hemoglobin production or total and individual cell volume.

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